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Single mucosal vaccination targeting nucleoprotein provides broad protection against two lineages of influenza B virus

Myung Hee Kim[a], Jung-Ok Kang[a], Joo-Young Kim[a], Hi Eun Jung[b], Heung Kyu Lee[b], Jun Chang[a,*]

[a] Graduate School of Pharmaceutical Sciences, Ewha Womans University, Seoul, Republic of Korea
[b] Biomedical Science and Engineering Interdisciplinary Program, Korea Advanced Institute of Science and Technology (KAIST), Daejeon, Republic of Korea

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ABSTRACT

Nucleoprotein is highly conserved among each type of influenza viruses (A and B) and has received significant attention as a good target for universal influenza vaccine. In this study, we determined whether a recombinant adenovirus encoding nucleoprotein of type B influenza virus (rAd/B-NP) confers protection against influenza virus infection in mice. We also identified a cytotoxic T lymphocyte epitope in the nucleoprotein to determine B-NP-specific CD8 T-cell responses. We found that B-NP-specific CD8 T cells induced by rAd/B-NP immunization played a major role in protection following influenza B virus infection using CD8 knockout mice. To assess the effects of the administration routes on protective immunity, we immunized mice with rAd/B-NP via intranasal or intramuscular routes. Both groups showed strong NP-specific humoral and CD8 T-cell responses, but only intranasal immunization provided complete protection against both lineages of influenza B virus challenge. Intranasal but not intramuscular administration established resident memory CD8 T cells in the airway and lung parenchyma, which were required for efficient protection. Furthermore, rAd/B-NP in combination with rAd/A-NP protected mice against lethal infection with both influenza A and B viruses. These findings demonstrate that rAd/B-NP could be further developed as a universal vaccine against influenza.

1. Introduction

Influenza B viruses are respiratory pathogens that belong to the *Orthomyxoviridae* family and have an impact on human health by causing seasonal influenza in humans as well as influenza A viruses (McCullers et al., 2004; Ambrose and Levin, 2012). Influenza B viruses comprise two lineages, the B/Victoria and B/Yamagata lineages (Rota et al., 1990) and are found only in human populations and seals. Influenza B virus is less common and less frequently associated with severe disease and have little potential for pandemics because of a lack of animal reservoir (Jackson et al., 2011). However, several studies have reported that influenza B viruses establish lower respiratory tract infections, which induce acute respiratory distress syndrome (ARDS) (Li et al., 2008; Li et al., 2009; Gutiérrez-Pizarraya et al., 2012; Huang et al., 2011; Kelvin et al., 2014). Influenza B viruses occasionally predominate during seasonal influenza epidemics and two lineages of influenza B viruses frequently co-circulate (McCullers et al., 2004; Rota et al., 1990; Li et al., 2008; Olson et al., 2007; Hite et al., 2007). Despite these conditions, the currently used trivalent vaccines contain components of only one strain of two influenza B lineages. Because there is little or no cross-protection between the two influenza B virus lineages, its effectiveness might be reduced in seasons with a different predominant circulating influenza B virus lineage (Beran et al., 2009; Belshe, 2010) or when two influenza B lineages are co-circulating (Ambrose and Levin, 2012; Belshe, 2010; Heikkinen et al., 2014). To address this problem, a quadrivalent influenza vaccine has been introduced in several countries (Belshe, 2010; van de Sandt et al., 2015). These inactivated influenza vaccines aim to elicit production of virus-neutralizing antibody directed mainly against the variable globular head region of the HA glycoprotein (Fiore et al., 2009). Accordingly, this requires annual update of the components of influenza vaccines through seasonal influenza surveillance. Further, current influenza vaccine often offers poor protection against influenza virus strains with unpredictable antigenic drift or novel emerging subtypes. These limitations of current vaccines emphasize the need to develop a novel vaccine, a so-called “universal influenza vaccine”, that induces protective immunity to several influenza virus strains.

Previous studies suggest that conserved internal proteins such as nucleoprotein (NP) and matrix protein (M1) could be a good target antigen for universal influenza vaccine due to high degree of homology among various viruses (Ulmer et al., 1993; Lee et al., 2008; Furuya et al., 2010; Fontana et al., 2014; Baranowska et al., 2015). It was

* Corresponding author. Laboratory of Immunology, Graduate School of Pharmaceutical Sciences, Ewha Womans University, Seoul, 03760, South Korea.
E-mail address: tcell@ewha.ac.kr (J. Chang).

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shown that virus-specific cytotoxic T lymphocytes (CTL) are an important mediator of cross-protective immunity to a broad range of influenza viruses (van de Sandt et al., 2015; Hillaire et al., 2011a,b). Lung-resident memory (T_{RM}) CD4 and CD8 T cells, in particular, have been described to provide enhanced protection against heterologous influenza A challenge (Teijaro et al., 2011; Turner et al., 2014; Wu et al., 2014; Zens et al., 2016). Non-circulating T_{RM} cells are present in various tissues (e.g. skin, intestines, female reproductive tract, brain, and lung), which are identified by two key phenotypic markers, both CD103 and CD69, and provide effective protection against viral infections (Anderson et al., 2012; Mueller and Mackay, 2016; Jiang et al., 2012; Schenkel and Masopust, 2014; Shane and Klonowski, 2014). Furthermore, the route of initial priming is likely to influence the generation of CD8 T_{RM} cells in the lung (McMaster et al., 2018; Takamura et al., 2010). Hence, strategies that enable the establishment of lung T_{RM} cells to conserved epitopes will be essential for universal influenza vaccine.

We here investigated whether a recombinant adenovirus encoding NP of influenza B would provide mice with broad protection against influenza B infection. To this end, we generated rAd/B-NP encoding the full-length nucleoprotein derived from influenza virus B/Yamagata/16/88, and analyzed the protective efficacy by challenge with both B/Yamagata and B/Vitoria lineages (Kim et al., 2015). To analyze B-NP-specific CD8 T cells, we identified, to our knowledge for the first time, a dominant epitope of B-NP, which encompasses amino acid residues 166–174 and is restricted to MHC class I H-2D^{d}. We show that CD8 T cells, especially CD8 T_{RM} in the lungs are necessary for protection, and the mucosal immunization, not systemic immunization, establishes T_{RM} in the lung and leads complete protection against influenza B virus infection.

2. Materials and Methods

2.1. Ethics statement

The animal studies were approved by the Institutional Animal Care and Use Committees (IACUCs) of Ewha Womans University (Approval No. 14–083 and 18-005) and the KAIST (Approval No. KA 2013-55). All animal experiments were performed in strict accordance with the animal handling policies mandated by the Guidelines for Animal Use and Care of the Korea Center for Disease Control (K-CDC).

2.2. Mice and virus strains

Female BALB/c and C57BL/6 mice (5–6 weeks old) were obtained from Orient Bio (Seoul, Korea) and CD8 knockout (KO) (B6.129S2-*Cd8a*^{tm1Mak}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were bred and maintained in the specific pathogen-free (SPF) facility.

The mouse-adapted influenza virus strain B/Florida/04/06 (B/Florida, Yamagata lineage) was kindly provided by Dr. Young Ki Choi (Chungbuk National University, Cheongju, Korea) and used for challenges (Kim et al., 2015). The B/Yamagata/16/88 (B/Yamagata, Yamagata lineage) virus was provided by Dr. Man Ki Song (International Vaccine Institute, Seoul, Korea) and used for cloning of NP gene, ELISA and challenges. The B/Shangdong/7/97 (B/Shangdong, Victoria lineage) virus and A/Puerto Rico/8/34 (PR8) virus were provided by Dr. Baik Lin Seong (Yonsei University, Seoul, Korea) and used for ELISA and challenges. B/Yamagata/16/88 and B/Shangdong/7/97 were propagated in Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34, Manassas, VA), as described previously (Donis et al., 2014; Shin et al., 2015). Briefly, the culture supernatant of infected MDCK cells was cleared by centrifuging at 600 g for 10 min (1580GRM, Gyrozen, Daejeon, Korea) and centrifuged at 100,000 g for 1 h (Optima LE-80K Ultracentrifuge, Beckman Coulter, Brea, CA) to precipitate virus. The precipitated virus was suspended in phosphate buffered saline (PBS) and stored at −70 °C until needed. Virus titers were determined by using plaque assay. PR8 virus was prepared as described (Kim et al., 2013).

2.3. Cells

Human embryonic kidney 293 (HEK293) cells integrated with Ad5 DNA (ATCC CRL-1573) and MDCK cells were grown in minimal essential medium (MEM) (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum.

2.4. Construction of recombinant replication-defective adenoviruses

Viral RNA from B/Yamagata was extracted using an RNeasy Mini kit (Qiagen, Valencia, CA) following manufacturer's instructions. NP gene cDNA was generated by reverse transcription using a forward primer (5′-GGGTACCGCCACCATGTCCAACATGGAT-3′) that contains a *Kpn*I restriction enzyme site and the Kozak sequence to enhance translation, and amplified by polymerase chain reaction using the above forward primer and a reverse primer (5′-GAGATATCTCATTAATAGTCGAGGTC ATCATA-3′) that contains two stop codons and an *Eco*RV restriction enzyme site at 3′ terminus. The entire open reading frame was inserted into the pShuttle-CMV vector through *Kpn*I/*Eco*RV double digestion. For generation of replication-defective adenovirus (serotype 5), the NP sequence was inserted into the adenovirus genome through homologous recombination as described previously (He et al., 1998). Briefly, the shuttle vector plasmid was electroporated into competent BJ5183 cells carrying the pAdEasy-1 Ad5 genomic DNA. Recombinant Ad5 DNA was then isolated and transfected into HEK293 cells to generate rAd/B-NP. As a control, rAd/mock or rAd/Gcf adenovirus encoding the core fragment of respiratory syncytia virus (RSV) glycoprotein was used. Adenovirus encoding NP of PR8 (rAd/A-NP) is described elsewhere (Kim et al., 2013). The recombinant adenoviruses were amplified using HEK293 cells and purified by double cesium chloride gradient centrifugation.

2.5. Determination of CTL epitopes for influenza B nucleoprotein

2.5.1. Prediction of candidate CTL epitopes for influenza B virus nucleoprotein

Candidate CTL epitopes for B-NP were predicted using publically available prediction methods, such as BIMAS (http://www-bimas.cit.nih.gov/molbio/hla_bind), SYFPEITHI (http://www.syfpeithi.de), and IEDB (http://tools.iedb.org) (Table 1S). The selected candidate peptides with highest scores were synthesized without any modification (> 80% purity) (Peptron, Seoul, Korea).

2.5.2. Evaluation of synthetic peptides as candidate CTL epitopes

Single-cell suspensions were prepared from lungs of mice immunized with rAd/B-NP and challenged with B/Yamagata. Cells (1×10^6) were cultured with medium alone or 10 μg/ml synthetic peptides for 5 h in the presence of Brefeldin A. After 5 h, the cell populations were analyzed for CD44$^+$IFN-γ$^+$ CD8 T cells by flow cytometry.

2.5.3. In vivo cytotoxicity assay

Splenocytes were prepared from naïve mice and stained with 5 μM or 500 nM CFSE (eBioscience, San Diego, CA). Labeled cells were then pulsed with the indicated peptides (1 μg/ml) and transferred intravenously (IV) (2.5×10^6 cells of each population) into the indicated groups of mice. After 5 h, lymphocytes were isolated from the spleen. Target cells were distinguished from recipient cells based on CFSE staining. Percent specific lysis was calculated as follows:

$$Ratio = irrelevant\ percentage{:}\ epitope\ specific\ percentage\ ([low]peak : [high]peak)$$

$$Percent\ specific\ lysis = \left[1 - \frac{transferred\ control\ ratio}{experimental\ ratio}\right] \times 100$$

2.6. Vaccination and challenge

For vaccination, 6-week-old mice were inoculated with various doses of rAd/B-NP vaccine or control adenovirus (rAd/Gcf) through the intranasal (IN) or intramuscular (IM) route. For IN immunization, mice were lightly anesthetized with isoflurane (Ifran®; Hana Pharm, Kyonggi-Do, Korea), and 1×10^7 to 1×10^8 plaque-forming units (PFUs) of rAd/B-NP or 1×10^8 PFU control adenovirus in 50 µl of PBS were applied to the left nostril. Three weeks after immunization, mice were lightly anesthetized with isoflurane and challenged IN with $10 \times 50\%$ lethal dose (10 LD_{50}) of B/Florida (Kim et al., 2015), B/Yamagata or B/Shangdong/7/97 (not published). Occasionally, mice were inoculated with rAd/A-NP + rAd/B-NP and challenged with 10 LD_{50} of B/Yamagata or PR8. One LD_{50} of B/Yamagata was measured to be 5×10^4 PFUs for BALB/c mice.

2.7. ELISA

Blood was acquired from the retro-orbital plexus using a heparinized capillary tube, centrifuged, and serum stored at −70 °C. Serum antibody titers against NP were determined by ELISA. Briefly, for coating of antigens, B/Yamagata and B/Shangdong/7/97 were disrupted with 0.5% Triton X-100 (Sigma, St. Louis, MO). Next, 96-well flat-bottom plates (Thermo Scientific Nunc, Denmark) were coated with virus (3600 PFUs equivalent/well) and then blocked with PBS containing 1% non-fat milk and 0.05% Tween 20. Serial dilutions of serum were added and incubated for 2 h. After washing, HRP-conjugated rabbit anti-mouse IgG (Abcam, UK) was used as a secondary antibody. Color development was analyzed at 450 nm using a Thermo Multiskan® EX (Vantaa, Finland).

2.8. In vivo labeling and tissue harvest

For *in vivo* antibody labeling, immunized mice were intravenously injected with 3 µg of APC-conjugated anti-CD45 antibody (30-F11; BioLegend, San Diego, CA) in 200 µl PBS and sacrificed 5 min later.

Bronchoalveloar lavage (BAL) was harvested by washing the airways with 1 ml of PBS. BAL cells were separated via centrifugation. Lung tissues were homogenized and passed through a 70 µm cell strainer (SPL). The parenchymal cells were isolated by digestion in Collagenase type II (Worthington) and DNase I (Sigma) (1 mg/ml Collagenase type II and 0.1% DNase I in RPMI1640 supplemented with 10% FBS) for 30 min at 37 °C. Spleens were mechanically dissociated into single-cell suspensions. Erythrocytes were removed using Red Blood Cell Lysing Buffer (Sigma).

2.9. Flow cytometric analysis

$D^d/NP_{166\text{-}174}$(FSPIRITFL) tetramer was produced as described previously (Altman et al., 1996). Briefly, recombinant proteins were expressed in *Escherichia coli*, purified from inclusion bodies, solubilized, and refolded in the presence of $NP_{166\text{-}174}$ peptide. Folded complexes were purified by Superdex-75 gel filtration chromatography, then biotinylated with the biotin–protein ligase BirA and tetramerized with fluorochrome-labeled streptavidin (Molecular Probes). For cell phenotype analysis, the isolated cells were washed with FACS buffer (0.5% FBS, 0.1% NaN_3 in PBS), blocked with purified rat anti-mouse CD16/CD32 (BD Pharmingen, San Diego, CA) and 5 µg/ml streptavidin (Invitrogen), and stained with anti-CD8a (clone 53–6.7), anti-CD44 (clone IM7), anti-CD69 (clone H1.2F3), anti-CD103 (clone 2E7) (BD Pharmingen or BioLegend), and $D^d/NP_{166\text{-}174}$(FSPIRITFL) tetramer. After staining, the cells were fixed in FACS lysing solution (BD Biosciences, San Diego, CA). To identify dead cells, the cells were not fixed and stained with DAPI (BioLegend) for 15 min at RT before running on the flow cytometer. To evaluate the cytokine-producing cells, mouse lung-derived lymphocytes were stimulated with 10 µM B-$NP_{166\text{-}174}$ (FSPIRITFL) peptide for 5 h in the presence of Brefeldin A (eBioscience) at 37 °C. After stimulation, these cells were then stained with surface markers, fixed, permeabilized with FACS buffer containing 0.5% saponin (Sigma), and stained with anti-mouse IFN-γ (clone XMG1.2; BioLegend). Stained cells were acquired using a FACSCalibur flow cytometer (BD Biosciences) and a BD LSR Fortessa (BD biosciences), and analyzed using the Flowjo software (TreeStar Inc., Ashland, OR).

2.10. Statistical analysis

All data are presented as means ± SEM (n = 4–7), and were compared by unpaired, two-tailed Student's *t*-test, one-way ANOVA, and two-way ANOVA, respectively. The results were followed up by post hoc analysis using Bonferroni's procedure and the differences were considered statistically significant when P values were < 0.05.

3. Results

3.1. Generation and characterization of recombinant adenovirus encoding the NP of influenza B virus

The complete coding sequence of the NP gene was cloned into the shuttle vector under the control of the cytomegalovirus (CMV) promoter, yielding pShuttle-CMV/B-NP, and then inserted into early region 1 (E1) of the adenovirus genome by homologous recombination, resulting in generation of replication-defective rAd/B-NP (Fig. S1A). To confirm the expression of the NP in rAd/B-NP recombinant adenovirus, HEK293 cells were infected with rAd/B-NP and immunoblot analysis of the infected cell lysates was conducted using a B/Yamagata-specific polyclonal antibody. We detected a single band at an approximate molecular weight of 61.6 kDa representing B-NP in rAd/B-NP-infected cell lysates. However, no such band was detected in uninfected or rAd/mock-infected cell lysates used as negative controls (Fig. S1B).

To examine the B-NP-specific CD8 T-cell responses, candidate CTL epitopes were predicted using publically available prediction methods, BIMAS, SYFPEIFHI, and IEDB (Table 1S). Synthetic peptides identified as candidate influenza B-NP epitopes in the context of $H\text{-}2^d$ were evaluated in terms of their ability to stimulate memory CD8 T cells of mice immunized with rAd/B-NP and then challenged with B/Yamagata. As shown in Fig. S2A, of eight candidate peptides, only B-$NP_{166\text{-}174}$ (FSPIRITFL) stimulated significant numbers of memory CD8 T cells. Because B-$NP_{166\text{-}174}$ was predicted to bind to $H\text{-}2D^d$, we next generated D^d/B-$NP_{166\text{-}174}$ tetramer. Lung lymphocytes from immunized mice were then stained with D^d/B-$NP_{166\text{-}174}$ tetramer-PE. Fig. S2B shows that D^d/B-$NP_{166\text{-}174}$ tetramer stained lung lymphocytes from rAd/B-NP-immunized mice specifically, but not those from rAd/mock-immunized mice. In contrast, irrelevant K^d/RSV M2 tetramer stained neither lung lymphocytes from rAd/mock-immunized nor rAd/B-NP-immunized mice, suggesting that D^d/B-$NP_{166\text{-}174}$ tetramer binds specifically to B-NP-specific memory CD8 T cells. These results indicate that B-$NP_{166\text{-}174}$ is an immunodominant CTL epitope of B-NP.

3.2. Humoral and cytotoxic T-cell responses induced by mucosal rAd/B-NP immunization

To investigate the influenza virus NP-specific immune responses induced by mucosal rAd/B-NP vaccination, BALB/c mice were inoculated intranasally (IN) with 1×10^8 PFUs of rAd/B-NP (designated B-NP hereafter). Also, mice were immunized IN with 1×10^8 PFUs of

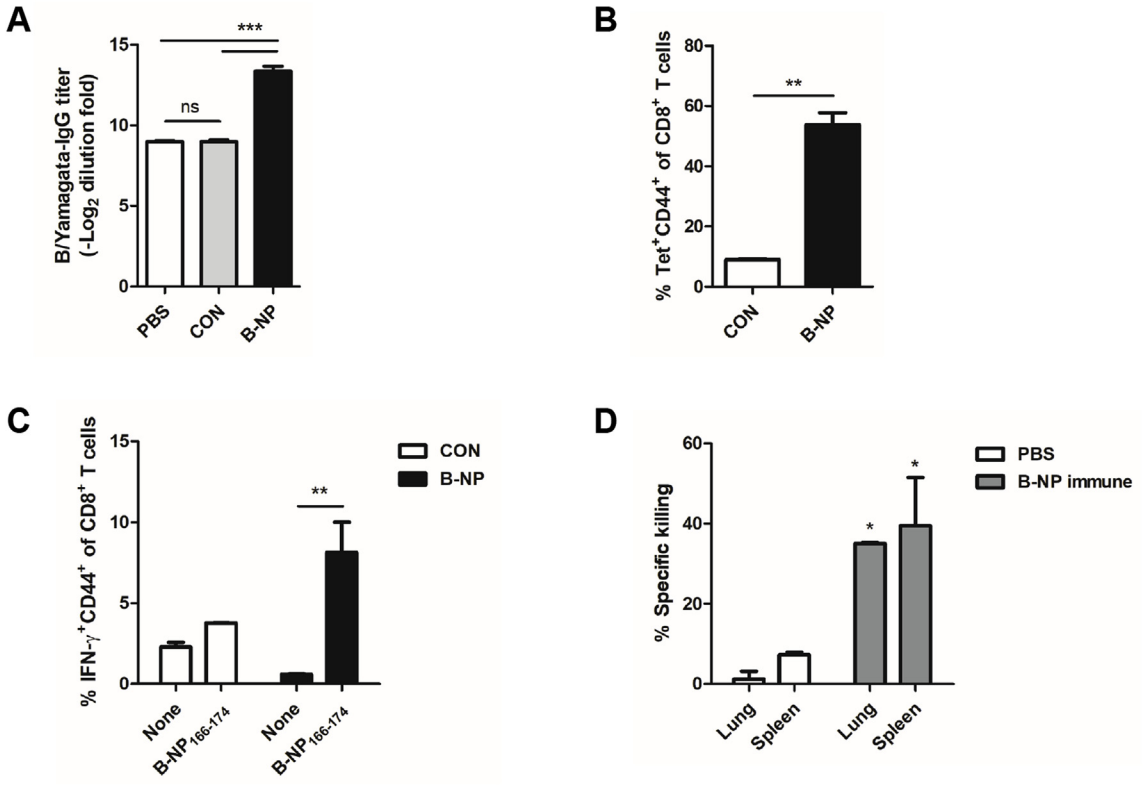

Fig. 1. Evaluation of NP-specific IgG in serum and NP-specific CD8 T cells in the lungs of B-NP-immune mice. BALB/c mice (n = 4 per group) were vaccinated with 1×10^8 PFUs of rAd/B-NP (B-NP) via the intranasal route, whereas control animals received PBS or 1×10^8 PFUs of rAd/Gcf (CON). At 3 weeks after vaccination, mice were challenged with 10 LD_{50} of B/Florida/4/06 virus. (A) Anti-NP IgG titers were determined by ELISA on day 14 after vaccination. (B) The average percentages of $D^d/NP_{166\text{-}174}$ tetramer-specific CD8 T cells at 7 day post-challenge. Lung cells were harvested from mice and stained with $D^d/NP_{166\text{-}174}$ tetramer, anti-CD8, and anti-CD44 antibody. (C) The average percentages of IFN-γ-expressing CD8 T cells in the lungs. Lung cells were also unstimulated or stimulated with $NP_{166\text{-}174}$ peptide in the presence of Brefeldin A for 5 h and then stained with anti-CD8, anti-CD44 and anti-IFN-γ antibody. (D) Percent killing was determined by *in vivo* cytotoxicity assay. Mice were immunized with 1×10^7 PFUs of B-NP and challenged with 1×10^5 PFUs of B/Yamagata 3 weeks after immunization. Other mice were administered only PBS. At 6 days post-challenge, target cells were transferred into challenged mice or unchallenged PBS mice and the lungs and spleens of the mice were harvested at 5 h after adoptive transfer. Data are the representative of at least two independent experiments with similar results and average SEM of four mice. ***$p^{<}0.001$; **$p^{<}0.01$; *$p^{<}0.05$.

irrelevant rAd virus, rAd/Gcf, as a control. Two weeks later, sera were harvested from all immunized mice, and the levels of B/Yamagata-specific IgG in the immune sera were determined via ELISA using detergent-disrupted B/Yamagata/16/88 virus as the coating antigen. Vaccination groups that received B-NP exhibited significantly higher levels of B/Yamagata-specific serum IgG compared to the control groups (Fig. 1A).

We also determined the ability of B-NP vaccination to induce specific CD8 T-cell responses by analyzing NP-specific CD8 T cells in the lungs following vaccination and subsequent influenza B challenge. The mice immunized IN with the control adenovirus or B-NP were challenged with lethal dose of B/Florida/4/06 virus (10 LD_{50}) at 3 weeks after immunization. At day 7 post-challenge, lung lymphocytes were harvested and the frequencies of $D^d/NP_{166\text{-}174}$ tetramer-specific CD8 T cells were determined via flow cytometry. As shown in Fig. 1B, B-NP vaccination resulted in the massive recruitment of $D^d/NP_{166\text{-}174}$ tetramer-specific CD8 T cells in the lungs compared to the control. NP-specific IFN-γ-secreting CD8 T cells were also increased significantly in B-NP-vaccinated mice (Fig. 1C).

Next, we performed *in vivo* cytotoxicity assays to examine killing function of B-$NP_{166\text{-}174}$-specific CD8 T cells. The antigen-specific killing efficacy of CD8 T cells is important for protective immunity against infection. Experimental mice received B-$NP_{166\text{-}174}$ peptide-pulsed target cells ($CFSE^{hi}$) mixed with irrelevant $OVA_{257\text{-}264}$ peptide -pulsed cells ($CFSE^{lo}$) in 1:1 ratio. Fig. 1D shows that B-NP-immune mice exhibited specific killing of B-$NP_{166\text{-}174}$ peptide-pulsed $CFSE^{hi}$ target cells in the lungs and spleens. Taken together, these results indicate that immunization with B-NP via IN route elicits humoral immune responses as well as cytotoxic CD8 T-cell responses.

3.3. Protection against homologous and heterologous virus challenge by mucosal B-NP immunization

Previously, we showed that B-NP vaccination induced B/Yamagata-specific IgG and B-$NP_{166\text{-}174}$-specific CD8 T-cell responses. Next, we investigated whether B-NP vaccination provided protection against homologous and heterologous influenza B virus infections. To this end, BALB/c mice were immunized, challenged with a lethal dose of B/Florida or heterologous B/Shangdong/7/97 virus after 3 weeks, and then monitored for 10–14 days to determine morbidity and mortality. As shown in Fig. 2A and B, all the control groups showed significant weight losses and succumbed to infection, while B-NP-immunized mice exhibited complete protection against lethal homologous B/Florida challenge. For heterologous B/Shangdong/7/97 virus challenge, all B-NP immune mice showed no weight loss and no mortality compared to the control mice, indicating complete protection against heterologous virus challenge (Fig. 2C and D). Interestingly, the 6th residue in the $NP_{166\text{-}174}$ sequence of B/Shangdong/7/97 virus is valine instead of isoleucine, and B-NP vaccination still confers protection against CTL epitope-variant virus challenge. Together, these results suggest that B-NP vaccination can provide protection against infection not only with homologous but also different lineages of influenza B virus.

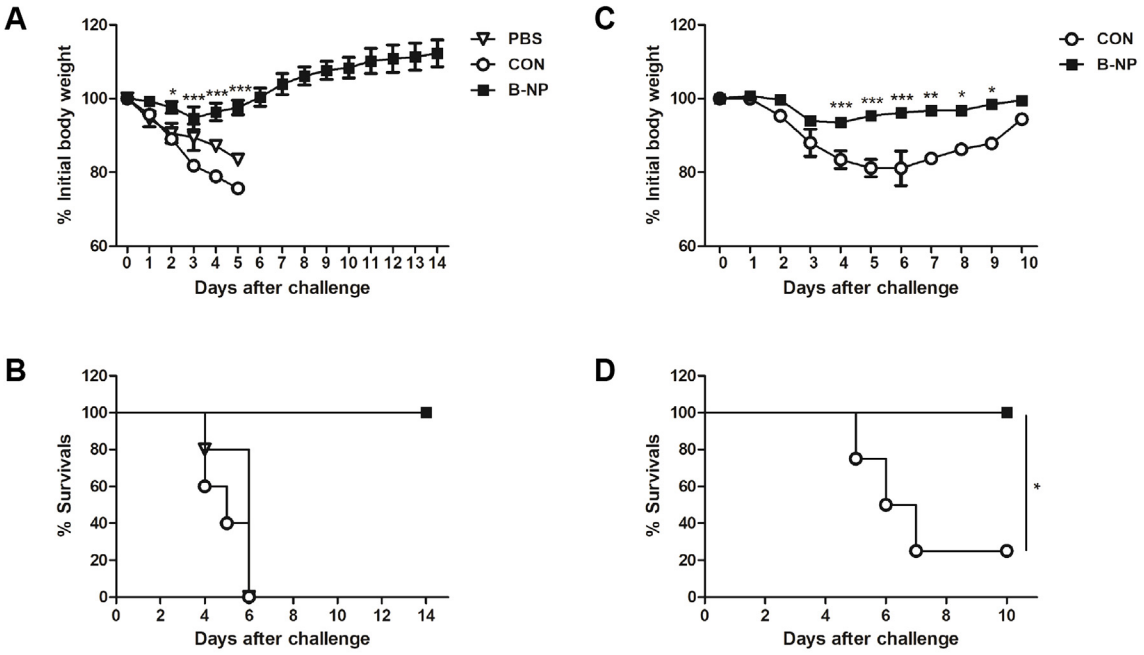

Fig. 2. Protective efficacy of B-NP vaccination against homologous and heterologous influenza B virus challenge. (A–B) BALB/c mice (n = 5 per group) were immunized with 1×10^8 PFUs of rAd/B-NP (B-NP) via the intranasal route. After 3 weeks, the mice were challenged with 10 LD_{50} of B/Florida/4/06 virus. (A) Body weight ($^*P < 0.05$ and $^{***}P < 0.001$, B-NP group compared to CON group) and (B) survival rate ($P < 0.01$, B-NP group compared to CON group) were recorded daily for 14 days. (C–D) BALB/c mice (n = 4 per group) were immunized with 1×10^8 PFUs of B-NP by the intranasal route. After 3 weeks, the mice were challenged with 10 LD_{50} of B/Shangdong/7/97 virus. (C) Body weight ($^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, B-NP group compared to CON group) and (D) survival rate ($^*P < 0.05$, B-NP group compared to CON group) were recorded daily for 10 days. Statistical difference in (A) and (C) was analyzed by Two-way ANOVA, and the values are expressed as mean ± SEM. Statistical analysis (Log-rank Test) in (B) and (D) was performed using GraphPad prism 5. Data are the representative of two independent experiments with similar results and average SEM of five mice in (A) and four mice in (C).

3.4. CD8 T cells play a critical role in protection against influenza B virus infection

Cytotoxic CD8 T lymphocyte (CTL)-mediated immunity targeting the conserved internal viral protein plays important effector roles in protection against lethal influenza A infection (Hillaire et al., 2011a,b). To elucidate a protective role of CD8 T cells in influenza B infection, both CD8 knockout (KO) and wild-type (WT) C57BL/6 mice were vaccinated IN with 1×10^8 PFUs of B-NP. Two weeks after immunization, sera were harvested and assessed for the levels of B/Yamagata-specific IgG by ELISA. CD8 KO mice generated B/Yamagata-specific serum IgG in equivalent titer to WT mice (Fig. 3A). Then, the mice groups were challenged with lethal doses of B/Yamagata virus (10 LD_{50}), and monitored for morbidity and mortality. The vaccinated CD8 KO mice lost body weight significantly until 6 dpi, and some begun to recover body weight gradually from 7 dpi, while WT mice did not exhibit significant weight loss (Fig. 3B). Importantly, CD8 KO mice showed significantly reduced survival rate (20%) compared to WT mice (100%; Fig. 3C). These results demonstrate that specific CD8 T cells induced by B-NP immunization are required to mediate complete protection from lethal influenza B virus challenge.

3.5. B-NP immunization via intranasal route rather than intramuscular route is important to establish protective immunity against influenza B virus

The route of immunization is thought to be important to generate protective immunity against influenza infection. Notably, mucosal immunization can protect more effectively from pulmonary infection than other routes (Belyakov and Ahlers, 2009). To assess the effects of the routes of administration on the protection against influenza B virus infection, BALB/c mice were inoculated IN or intramuscularly (IM) with 1×10^8 PFUs of B-NP. As a control, mice were immunized IN with 1×10^8 PFUs of rAd/mock. After 3 weeks, all mice were challenged with 10 LD_{50} of B/Yamagata. Mice that received B-NP via IM route as well as the control IN group succumbed to B/Yamagata infection by day 6. However, all the mice that received B-NP via IN route showed little body weight loss and no signs of disease and protected perfectly against lethal challenge (Fig. 4A and B). Interestingly, however, the percentage of D^d/B-$NP_{166\text{-}174}$ tetramer-specific CD8 T cells in the lungs of mice that received B-NP via either IN or IM route at 7 day post-challenge was detected at similar levels (Fig. 4C and D). These results suggest that B-$NP_{166\text{-}174}$-specific CTLs elicited by IM immunization are successfully recalled upon B/Yamagata challenge, but failed to protect the mice. Only IN immunization with B-NP provided complete protection against lethal B/Yamagata infection. Taken together, these results demonstrate that IN but not IM immunization with B-NP has generated protective immunity against influenza B virus infection.

3.6. Intranasal but not intramuscular immunization of B-NP establishes CD8 T_{RM} cells in the airway and lung parenchyma

Recent studies suggest that lung-resident memory CD8 T cells (T_{RM}) mediate protection against pulmonary infection, and inoculation via IN route is necessary to establish antigen-specific T_{RM} cells in the lung (McMaster et al., 2018; Takamura et al., 2010, 2016; Schmidt et al., 2018). We hypothesized that the protection by IN but not IM immunization with B-NP could be associated with T_{RM} generation. Thus, we investigated whether IN or IM immunization affects establishment of CD8 T_{RM} cells in the airway and lung parenchyma. To this end, BALB/c mice were vaccinated IN or IM with 1×10^8 PFUs of B-NP. All IN and IM immunization groups exhibited the similar levels of B/Yamagata-specific IgG at 2 weeks after immunization (Fig. S3A). B-$NP_{166\text{-}174}$-specific CD8 T_{RM} were enumerated in the lungs and spleens at 3 weeks post-vaccination. To distinguish between lung-localized and circulating T cells, mice were intravenously (IV) injected with anti-CD45-APC Ab. Cells that stained with anti-CD45 Ab were identified as

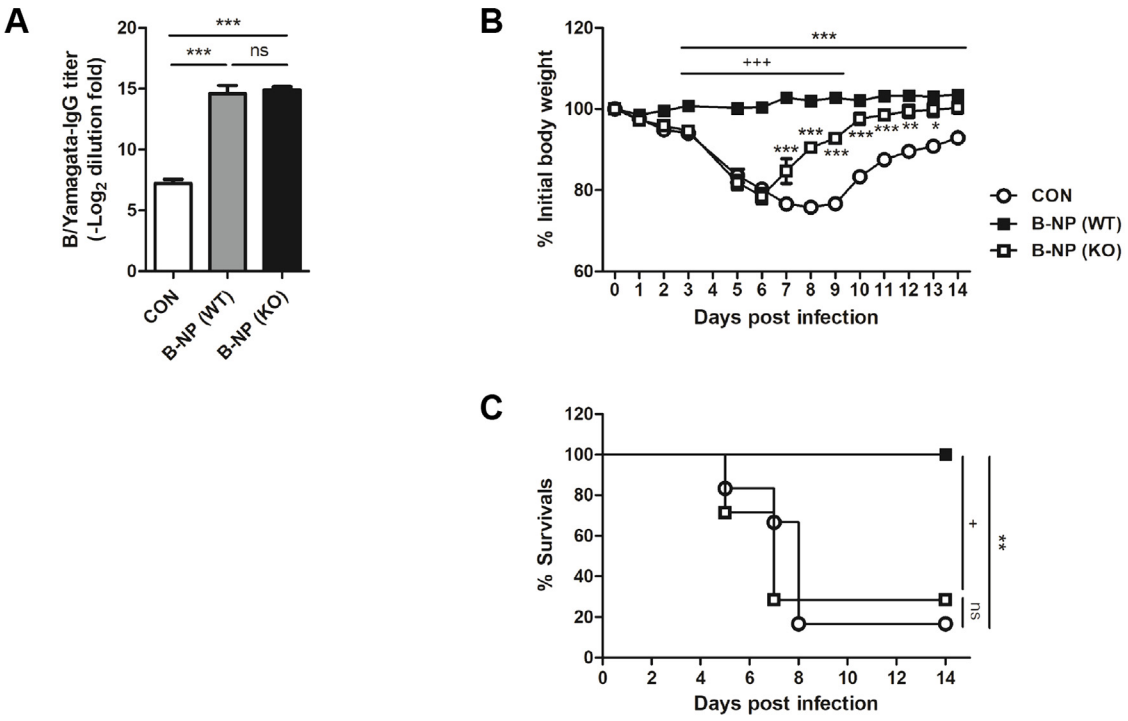

Fig. 3. Effects of deficiency of CD8 T cells on protective efficacy against B/Yamagata virus challenge. CD8 knockout (KO) and wild-type (WT) C57BL/6 mice (n = 6–7 per group) were IN immunized with 1×10^8 PFUs of rAd/B-NP (B-NP). As negative control, WT C57BL/6 mice were IN immunized with 1×10^8 PFUs of rAd/mock (CON). After 3 weeks, the mice were challenged with 10 LD_{50} of B/Yamagata/16/88 virus. (A) Anti-B-NP IgG titers were determined by ELISA on day 14 after vaccination. (B) Body weight and (C) survival rate were recorded daily for 14 days after challenge. Data are expressed in means ± SEM (n = 6–7/group). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, B-NP group compared to CON group; + $P < 0.05$ and + + + $P < 0.001$, B-NP group compared to B-NP (KO) group.

circulating T cells in the blood, and cells that were protected from *in vivo* labeling as lung-localized T cells (Fig. S3B, Fig. 5A). In the airway and lung parenchyma, the absolute numbers of $D^d/NP_{166\text{-}174}$ tetramer-specific CD8 T cells were significantly higher in IN immunized mice than IM immunized mice. However, the numbers of $D^d/NP_{166\text{-}174}$ tetramer-specific CD8 T cells in the lung vasculature were higher in IM group than IN group, suggesting that circulating memory T cells play relatively little role on protection (Fig. 5B). There were no significant differences in the numbers of $D^d/NP_{166\text{-}174}$ tetramer-specific CD8 T cells in the spleen between IN and IM vaccination groups (Data not shown). We next assessed whether B-$NP_{166\text{-}174}$-specific CD8 T cells in the airway and lung parenchyma express CD69 and CD103, the canonical markers of T_{RM} cells in the lung. The frequencies and the absolute numbers of $CD69^+$ $CD103^+$ $D^d/NP_{166\text{-}174}$ tetramer-specific CD8 T cells in the airway and lung parenchyma in IN immunized mice was higher than IM immunized mice (Fig. 5C–E). These results indicate that IN immunization has the better ability to establish CD8 T_{RM} cells in airway and lung parenchyma, which provide protective immunity against influenza B infection.

3.7. Immunogenicity and protective efficacy of B-NP immunization combined with A-NP

Previously, we have developed the recombinant adenovirus vaccine encoding A-NP and reported the possibility of universal protection against broad spectrum of influenza A viruses (Kim et al., 2013). To investigate whether combined immunization with A-NP and B-NP could provide simultaneous protection against both influenza A and B, mice were immunized with A-NP and B-NP (1×10^8 PFUs per each vaccine) or each separately, and challenged with 10 LD_{50} of either influenza A (PR8) or influenza B (B/Yamagata) at 3 weeks after immunization. Fig. 6A shows that the mice immunized with A-NP + B-NP vaccine as well as B-NP alone showed complete protection against lethal influenza B challenge. We measured CTL responses against B-NP at 5 dpi, and the levels of $D^d/NP_{166\text{-}174}$ tetramer-specific CD8 T cells in the lungs of A-NP + B-NP group were significantly reduced compared to those of the B-NP group (Fig. 6B), suggesting possible epitope dominance hierarchy and/or competition between A-NP and B-NP. A-NP + B-NP immunization as well as A-NP alone also provided complete protection against influenza A (PR8) challenge (Fig. 6C). The levels of A-NP-specific CD8 T cells in the lungs of A-NP + B-NP group were reduced compared to those of the A-NP alone group (Fig. 6D), also suggesting possible epitope dominance hierarchy and/or competition between A-NP and B-NP. Overall, our results suggest that immunization with A-NP + B-NP vaccine can confer broad protection against both influenza A and B viruses.

4. Discussion

The induction of cross-protective CD8 T cells, specific for conserved internal protein, could be a very efficient approach in the development of a universal influenza vaccine. In particular, vaccination strategy to induce cross-reactive NP-specific CD8 T cells has been considered for the development of broadly protective influenza A virus vaccines (Kim et al., 2013; Hillaire et al., 2011a,b; Yewdell et al., 1985). In the present study, we engineered a replication-defective recombinant adenovirus expressing full-length B-NP derived from B/Yamagata and investigated whether the priming immune response induced by the vaccine candidate could offer cross-protection against lethal homologous and heterologous influenza B challenge. Given that strong humoral and cellular immune responses are induced by intranasal immunization of rAd, even in the presence of pre-existing immunity to the adenovirus vector (Kim et al., 2013; Van Kampen et al., 2005; Croyle et al., 2008) and vaccination via the same route as pathogen invasion induces tissue-resident

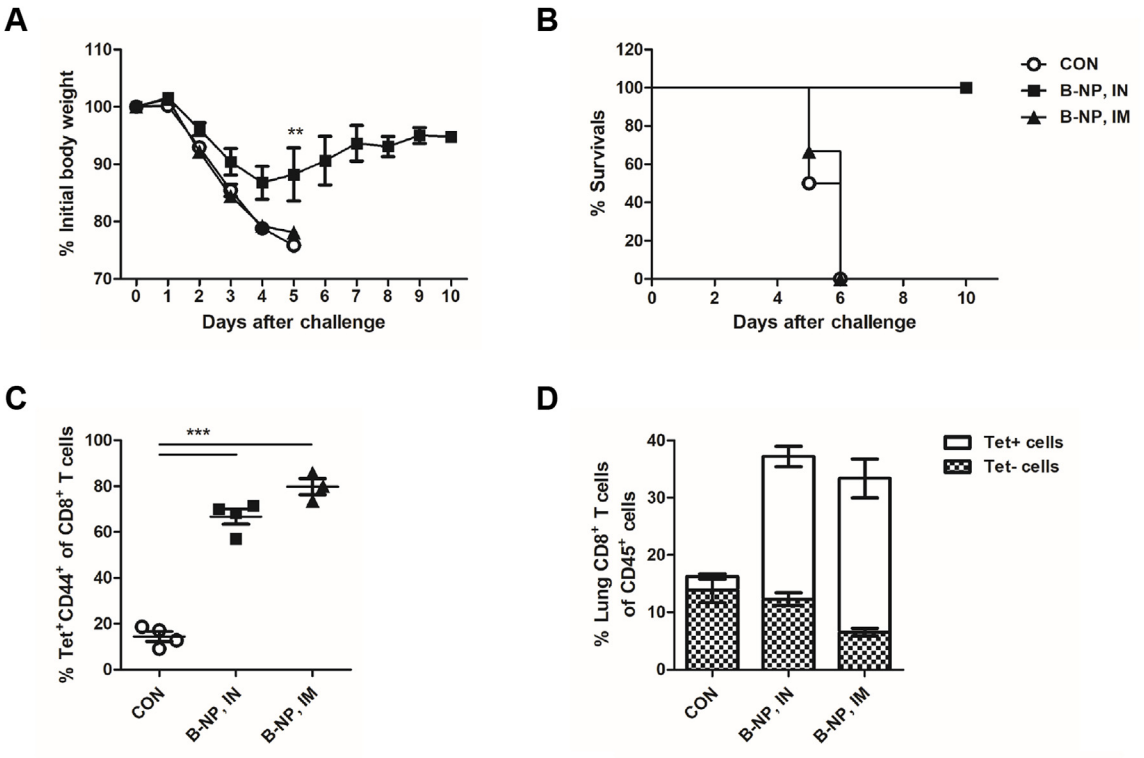

Fig. 4. Comparison of protective immunity in B-NP immunized mice via IN or IM route. BALB/c mice (n = 4 per group) were vaccinated via intranasal or intramuscular routes with 1×10^8 PFUs of rAd/B-NP (B-NP). Control mice were immunized intranasally with 1×10^8 PFUs of rAd/mock (CON). At 3 weeks after immunization, mice were challenged with 10 LD_{50} B/Yamagata/16/88 virus. (A) Body weight (**$P < 0.01$, IN group compared to IM group) and (B) survival rate ($P < 0.05$, IN group compared to IM group) were recorded daily for 10 days. At day 7 or 10 post-challenge, lung cells were harvested from mice and stained with D^d/$NP_{166\text{-}174}$ tetramer, anti-CD8, and anti-CD44 antibody. (C) The average percentages of D^d/$NP_{166\text{-}174}$ tetramer-specific CD8 T cells in the lung of four mice per group. (D) The frequency of tetramer-positive CD8 T cells and tetramer-negative CD8 T cells of $CD45^+$ cells in the lung. Data are the representative of two independent experiments with similar results and average SEM of four mice. ***$p^{<}0.001$.

memory T cells (Hogan et al., 2001; Turner and Farber, 2014; McMaster et al., 2015; Park and Kupper, 2015), the mice were immunized intranasally with our vaccine candidate. To analyze the CTL responses to the vaccine candidate, we identified a novel CTL epitope in B-NP. Our data demonstrated that the H-$2D^d$-restricted $NP_{166\text{-}174}$ peptide is the immunodominant CTL epitope to B-NP. Moreover, we revealed that intranasal immunization of B-NP induced significant frequency of B-NP-tetramer-specific and B-NP-specific IFN-γ-producing CD8 T cells in the lungs of B-NP-immunized mice after challenge with homologous B influenza virus. The immunization of B-NP also increased the B-NP-specific IgG level in serum. Mucosal immunization of B-NP provided complete protection against lethal challenge with both B/Yamagata-lineage and heterologous B/Victoria lineage virus. Using CD8 KO mice, we further showed that CD8 T cells mainly contribute to protection upon influenza B virus infection.

The development of immune responses at a site of viral exposure is necessarily correlated with host protection (Belyakov and Ahlers, 2009). In our study, it has been shown that the route of immunization is important for conferring protection against lethal influenza B infection. We compared the immune response to IN and IM immunization of B-NP. The B-NP-specific CD8 T-cell responses and B-NP-specific IgG levels were comparable between the two groups. However, IM immunization with B-NP failed to confer complete protection against B/Yamagata challenge, indicating protective efficacy depends on the route of immunization. Our explanation for the lack of protection against B/Yamagata, although there were B-NP-specific CD8 T cells induced by B-NP immunization via IM route, may be related to the establishment of T_{RM} cells in the lung. It is thought that tissue-resident T_{RM} cells mediate effective protective immunity against localized infections (Gebhardt et al., 2009; Jiang et al., 2012). T_{RM} cells can persist at frontline sites of pathogen entry, respond rapidly to the pathogen challenge, and provide protection independently of recruitment of circulating memory T cells (Schenkel et al., 2013). Even though the exact mechanisms of immediate protection mediated by the T_{RM} is not clear yet, it might be involved with efficient control of virus through rapid production of cytokines and chemokines and/or efficient killing of newly infected epithelial cells (Wu et al., 2014). Following respiratory virus infection, virus-specific T_{RM} cells are established in the lung airway and pulmonary tissues (Wu et al., 2014; Takamura et al., 2016; Schmidt et al., 2018). In the case of active immunization, it is likely that establishment of lung T_{RM} depends on the route of immunization (McMaster et al., 2018; Takamura et al., 2010). We observed that local (IN) immunization of B-NP generated tissue-resident CD8 T cells in the airway and lung parenchyma, and resulted in superior protection than systemic (IM) immunization. Mucosal immunization to the sites of pathogen entry might be necessary to establish specific lung T_{RM} cells that mediate protection against pulmonary infection (Rosato et al., 2017; Gilchuk et al., 2016; Wu et al., 2014; Zens et al., 2016), though the requirements for generation of tissue-resident T_{RM} cells are not clear yet. Our results are consistent with other reports that T_{RM} in the lungs of mice could be elicited by mucosal vaccination, but not systemic immunization (Wang et al., 2004; Morabito et al., 2017; Zens et al., 2016; McMaster et al., 2018). Airway and lung CD8 T_{RM} cells generated by mucosal administration maintain long-term protective immunity and provide enhanced heterosubtypic protection against respiratory virus infection compared to circulating T cells (McMaster et al., 2015; Teijaro et al., 2011; Wu et al., 2014). The immunization of mice with B-NP also dramatically increases antigen-specific IgG levels in the serum independent of the injection routes. However, IN immunized mice but not IM immunized mice exhibited substantial levels of NP-specific

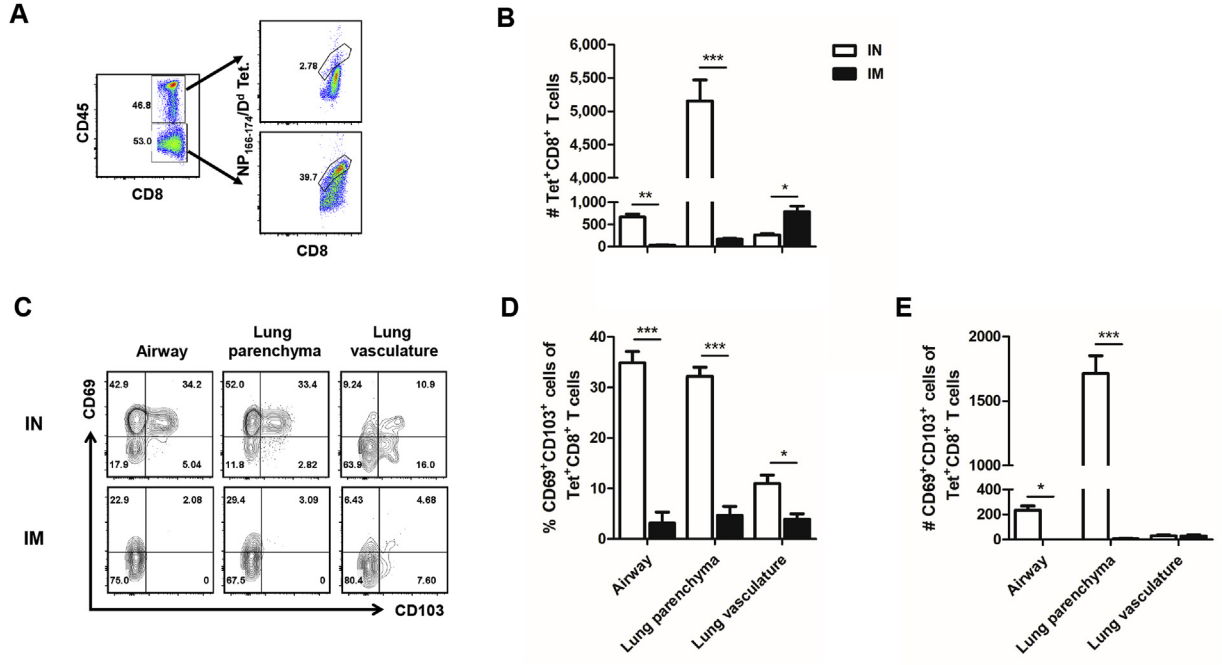

Fig. 5. Generation CD8 T_{RM} cells in the airway and lung parenchyma by IN immunization but not IM immunization. At 3 week after immunization with 1×10^8 PFUs of rAd/B-NP via the IN or IM route, mice were injected IV with 3 μg of anti-CD45 antibody and sacrificed 5 min later. (A) Representative IV staining used to identify cells in the vascular ($CD45^+$) and lung-resident ($CD45^-$) of B-$NP_{166-174}$-specific CD8 T cells. (B) The absolute number of $D^d/NP_{166-174}$ tetramer-specific CD8 T cells in the lung airway, lung parenchyma, and lung vasculature of five mice per group. (C) Expression of CD69 and CD103 on $D^d/NP_{166-174}$ tetramer-specific CD8 T cells in the lung airway, lung parenchyma, and lung vasculature of mice at 3 weeks after IN or IM immunization. (D) The average percentages and (E) total number of $CD69^+$ $CD103^+$ $D^d/NP_{166-174}$ tetramer-specific CD8 T cells in the lung airway, lung parenchyma, and lung vasculature at 3 weeks after IN or IM immunization. Data are expressed in means ± SEM (n = 5/group). ***$p<0.001$; **$p<0.01$; *$p<0.05$.

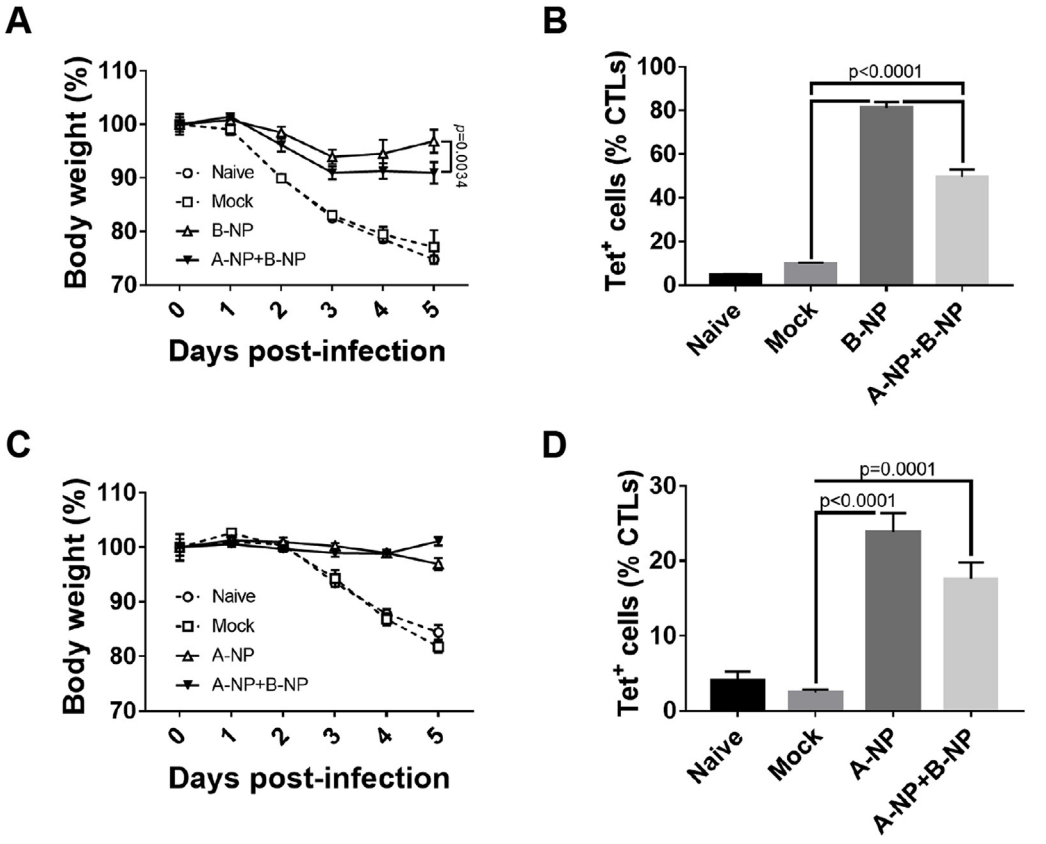

Fig. 6. Immunological and protective efficacy of rAd/B-NP vaccination combined with rAd/A-NP against both B/Yamagata and PR8 challenge. (A–B) BALB/c mice (n = 5 per group) were immunized with 2×10^8 PFUs of rAd/mock, rAd/B-NP or rAd/A-NP + rAd/B-NP by the intranasal route. Control mice were immunized intranasally with PBS or 1×10^8 PFUs of rAd/mock. After 3 wks, the mice were challenged with 10 LD_{50} of B/Yamagata/16/88. (A) Body weight was recorded daily for 5 days. (B) The average percentages of $D^d/NP_{166-174}$ tetramer-specific CD8 T cells in the lungs of five mice per group. At day 5 post-challenge, lung cells were harvested from mice and stained with $D^d/NP_{166-174}$ tetramer, anti-CD8, and anti-CD44 antibody. (C–D) BALB/c mice (n = 5 per group) were immunized with 2×10^8 PFUs of rAd/mock, rAd/A-NP or rAd/A-NP + rAd/B-NP by the intranasal route. Control mice were immunized intranasally with PBS or 1×10^8 PFUs of rAd/mock. After 3 wks, the mice were challenged with 10 LD_{50} of PR8. (C) Body weight was recorded daily for 5 days. (D) At day 5 post-challenge, lung cells were harvested from mice and stained with $K^d/NP_{147-155}$ tetramer, anti-CD8, and anti-CD44 antibody. The average percentages of $K^d/NP_{147-155}$ tetramer-specific CD8 T cells in the lungs of five mice per group. Data are the representative of two independent experiments with similar results and average SEM of five mice.

mucosal IgAs. Thus, it is possible that the presence of influenza NP-specific mucosal IgAs in the respiratory mucosa may be partly involved in the enhanced protection against the lethal influenza challenges.

In summary, our study demonstrates that single IN immunization of B-NP successfully establishes lung-resident CD8 T_{RM} cells and subsequently provides broadly protective immunity to both lineages of influenza B viruses, proposing that B-NP is a potential universal vaccine. To our knowledge, this study is the first to report development of an NP-targeting vaccine for influenza B viruses and identification of a CTL epitope in the NP of influenza B virus. Our B-NP vaccine candidate could be further developed as a universal vaccine in a composite vaccine regiment with A-NP vaccine against influenza A and B viruses.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.01.002.

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